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Award Number: DAMD17-00-1-0031

TITLE: Functional Analysis of C-CAM1 Tumor Suppressor Gene
        by Targeted Gene Deletions

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REPORT DATE: January 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
                        Distribution Unlimited

20030609 039

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE January 2003	3. REPORT TYPE AND DATES COVERED Final (1 Jan 00 – 31 Dec 02)

4. TITLE AND SUBTITLE
Functional Analysis of C-CAM1 Tumor Suppressor Gene by Targeted Gene Deletions

5. FUNDING NUMBERS
DAMD17-00-1-0031

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

C-CAM1 (renamed CEACAM1) is a cell adhesion molecule of the immunoglobulin supergene family. We have shown that CEACAM1 plays critical roles in prostate cancer initiation and progression. We propose to determine the functional roles of CEACAM1 in normal prostate development, prostate homeostasis and prostate tumorigenesis by using gene targeting technologies to generate CEACAM1 knockout mice. We have constructed a *Ceacam1* targeting vector in which *Ceacam1* gene was flanked by loxP sites to allow for generation of both straight and conditional knockout of the *Ceacam1* gene. Several embryonic stem cell clones containing the recombinant gene allele were established and two were injected into blastocysts for germ line transmission of the targeting construct. In this funding period, we have succeeded in producing mice harboring the conditional *Ceacam1* knockout allele. Two founder mice from two embryonic cell clones showed germ line transmission of the targeting construct containing the conditional *Ceacam1* gene. Five mice with homozygous conditional *Ceacam1* alleles were born from these two founder mice. These results suggest that embryonic lethality does not occur in the mice carrying conditional *Ceacam1* knockout alleles, thus it is possible to pursue the function of CEACAM1 in normal prostate development, prostate homeostasis and prostate tumorigenesis *in vivo*.

14. SUBJECT TERMS
Prostate Cancer

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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(4)   INTRODUCTION

C-CAM1 (recently renamed as CEACAM1) is a cell adhesion molecule of the immunoglobulin supergene family (1). We have shown that CEACAM1 plays critical roles in prostate cancer initiation and progression and that loss of CEACAM1 is an early event in the development of prostate cancer (2). Although tumorigenesis studies in mouse xenograft model have suggested the involvement of CEACAM1 in epithelial cell growth and differentiation, the functional roles of CEACAM1 in normal prostate development, prostate homeostasis, and prostate tumorigenesis remain unclear. Towards the aim of unraveling the roles of CEACAM1's growth suppressive activity in prostate growth and tumorigenesis, we propose to use gene targeting and embryonic stem cell technologies to generate CEACAM1 knockout mice. Specifically, we plan (1) to determine the roles of CEACAM1's growth suppressive function in vivo by generating mice with a targeted deletion of the CEACAM1 cytoplasmic domain; (2) to determine the roles of CEACAM1's growth suppressive function in prostate development and tumorigenesis by generating mice with a prostate-specific knockout of the CEACAM1 cytoplasmic domain. The proposed work was divided into two Tasks to be carried out in parallel.

Task 1. Generate mice with targeted deletion of CEACAM1 cytoplasmic domain to determine the roles of CEACAM1's growth suppressive function in vivo (months 1-30)

Task 2. Prostate-specific loss of function of CEACAM1 gene in prostate (months 7-36)

Genetic manipulation of mouse genes in vivo is a powerful approach to understanding the function of a gene, both during embryonic development and in adult tissues. This method requires full knowledge of the genomic structure of the gene of interest. Unlike humans and rats, which each have one CEACAM1 gene, two CEACAM1-like genes, *Ceacam1* and *Ceacam2*, were identified in mice. In the previous study, we have isolated and sequenced these two closely related *Ceacam* genes from a mouse 129 Sv/Ev library (3). We have also examined the tissue-specific and embryonic expressions of these mouse *Ceacam1* and *Ceacam2* genes (3). Our sequence analysis revealed that the genes encoded nine exons and spanned approx. 16-17 kb (*Ceacam1*) and 25 kb (*Ceacam2*). The genes were highly similar (79.6%). The major

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differences in the protein-coding regions were located in exons 2, 5 and 6. To determine whether functional redundancy exists between *Ceacam1* and *Ceacam2*, we examined their expression in 16 mouse tissues by using semi-quantitative reverse transcription-PCR. As in human and rat, in mouse *Ceacam1* mRNA was highly abundant in the liver, small intestine, prostate and spleen. In contrast, *Ceacam2* mRNA was only detected in kidney, testis and, to a lesser extent, spleen. In a mouse embryo, *Ceacam1* mRNA was detected at day 8.5, disappeared between days 9.5 and 12.5, and re-appeared at day 19. On the other hand, no *Ceacam2* mRNA was detected throughout embryonic development. The different tissue expression patterns and regulation during embryonic development suggest that the *Ceacam1* and *Ceacam2* proteins, although highly similar, may have different functions both during mouse development and in adulthood. Results from this study allow us to design a gene targeting strategy that is specific to *Ceacam1* gene and also allow us to perform both straight and conditioned knockout of *Ceacam1* gene in parallel.

We have designed a gene targeting strategy that is specific to *Ceacam1* gene. In addition, the *Ceacam1* gene in the targeting vector was flanked by loxP sites to allow for generation of both straight and conditional knockout of *Ceacam1* gene. The targeting vector has been constructed and 24 embryonic stem cell clones containing the recombinant gene allele have been established. Two of the embryonic cell clones were injected into blastocysts for germ line transmission of the targeting construct toward the end of last funding period. In this funding period, we have succeeded in producing mice harboring conditional *Ceacam1* gene. Two founder mice from two embryonic cell clones showed germ line transmission of the targeting construct. Five mice with homozygote conditional *Ceacam1* allele were born from these two founder mice. These results suggest that embryonic lethality does not occur in mice carrying conditional *Ceacam1* allele. Thus, it is possible to pursue the function of CEACAM1 in prostate in vivo.

(5) BODY (Progress report)

Because Task 1 and Task 2 are performed in parallel, we describe the progresses in these aspects together.

5. 1. Gene targeting strategy

Based on genomic characterization of *Ceacam1* gene, we decided to delete the cytoplasmic domain of CEACAM1. This strategy is based on the following rationales: (1) the entire *Ceacam1* gene, around 20 kb, is too large to delete; and (2) the cytoplasmic domain of CEACAM1 is critical for tumor suppression. A conditional knockout construct is designed to delete exons 7~9 in a tissue-specific or non-specific manner in the mice. ES cells harboring the targeting vector are injected into blastocysts to generate mice carrying the conditional *Ceacam1* allele. For straight knockout, the goal of Task 1, the conditional knockout mice will be crossed with transgenic mice carrying actin-driven Cre recombinase, which is expressed during embryonic development. For conditional knockout, the goal of Task 2, the *Ceacam1* gene will be deleted in prostate specifically by crossing the conditional knockout mice with transgenic mice carrying probasin-driven Cre recombinase. Thus, Task 1 and Task 2 are being performed in parallel. The gene knockout strategy is shown in Fig. 1.

Fig. 1. Maps of the *Ceacam1* genomic locus and the loxP-targeting vector designed to introduce a Neo^R cassette. Exons are denoted by rectangles and loxP sites are denoted by triangles. Red rectangles indicate CEACAM coding regions. Successful targeting yields a conditional Ceacam1^{floxed} allele. Cre-mediated recombination results in deletion of Neo^R cassette and the cytoplasmic domain exons, yielding a knockout allele. Specific primers used to screen for various alleles are indicated by small arrows labeled a through c.
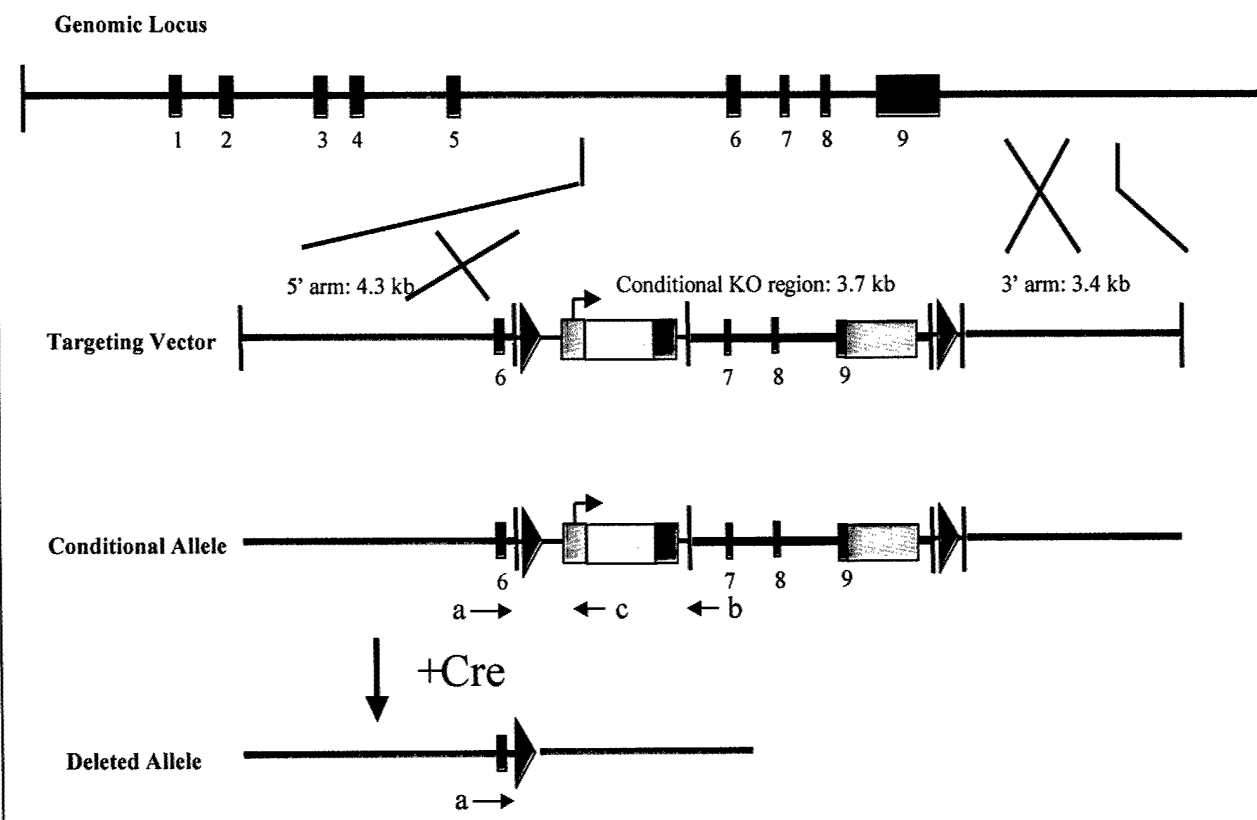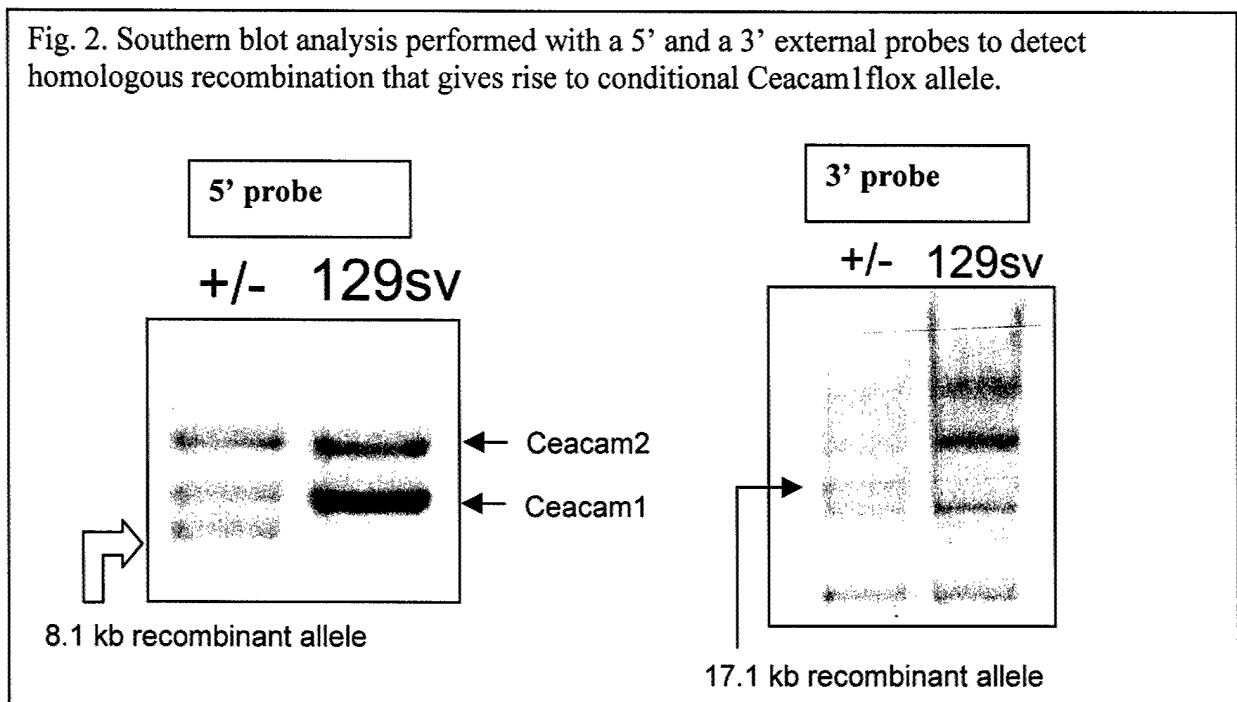
5. 2. Targeting vector construction

A 129Sv/Ev mouse genomic library in BAC vectors was screened with a probe generated from cDNA coding for the full-length mouse CEACAM1. Three positive BAC clones were identified and their structures were determined by restriction mapping. Consistent with our previous study (3), two closely related genes, i.e. *Ceacam1* and *Ceacam2*, were identified from restriction mapping. Based on tissue-specific and embryonic expression of *Ceacam1* and *Ceacam2* gene (3), we decided to delete the cytoplasmic domain of *Ceacam1*. A conditional knockout construct was designed to delete exons 7~9. The conditional targeting construct was designed by inserting a loxP-neo cassette at the Xmn1 site in intron 6 and a loxP fragment at the HindIII site in intron 9 of *Ceacam1* (Fig. 1). The targeting vector includes 4.3 kb of homologous DNA upstream of the loxP-neo site and 3.4 kb of homologous DNA downstream of the second loxP site. The targeting vector was verified by restriction mapping.

5. 3. Transfection and generation of embryonic stem cells harboring targeting vector

The targeting vector was transfected into embryonic stem cells by electroporation. A total of 270 ES cell clones were obtained from two electroporations. Genomic DNA was extracted from these ES cell clones. Half of the DNA from each sample was digested with SphI and processed for Southern blot analysis. The 5' probe used is a 0.7kb ApaI/SpeI fragment from subclone BamHI-A. Using the 5' probe, a ~9.2 kb band corresponding to the endogenous *Ceacam1* and a ~10 kb band corresponding to *Ceacam2* were detected in most samples. Many clones (a total of 24) included an additional hybridization signal at the size (8.1 kb) predicted for the homologous recombinant allele (Fig. 2). These potential positives clones were digested with Xho I and hybridized with the 3' probe, which is a 1.2kb Sac I/Xho I fragment from subclone Apa I-4. A ~15.3 kb band corresponding to the endogenous allele of Ceacam1 and a ~17.1 kb band corresponding to recombinant allele were detected in 16 clones (Fig. 2). These 16 clones were also detected with a Neo probe, which gave a 17.1 kb band in Southern blot analysis. Taken together, a total of 16 ES cell clones containing the *Ceacam1* conditional allele were generated. Three positive ES cell clones were injected into mouse blastocysts using procedures described in Chang et al. (4). Chimeric mice were obtained and they were mated with C57BL

mice. The F1 mice having germ line transmission of the loxP-targeted *Ceacam1* allele were interbred to generate F2 mice.

Fig. 2. Southern blot analysis performed with a 5' and a 3' external probes to detect homologous recombination that gives rise to conditional Ceacam1flox allele.

5' probe

3' probe

+/- 129sv

+/- 129sv

← Ceacam2

← Ceacam1

8.1 kb recombinant allele

17.1 kb recombinant allele

5. 4. Genotyping of the wild-type and loxP-targeted (floxed) alleles

Germ line transmission was confirmed by PCR analysis. Isolated mouse tail DNA was used for PCR identification of the genotype. Three sets of primers that detect the wild-type and the *floxed* allele of Ceacam1, and the Ceacam2 gene were used to genotype the mice. Primer A (5' ACACAAGGAGGCCTCTCAGATGGCG 3') and Primer C (5'GCGCCTCCCCTACCCGGTAGAATT 3'), containing sequences from Exon 6 of *Ceacam1* and neomycin cassette, respectively, will produce a 488 bp PCR product from the *floxed Ceacam1* allele. Primer A and Primer B (5' GACTTTGGCTTCCTGACTGGAGGA 3'), containing sequences from Intron 6, will generate a 382 bp PCR product from wild-type *Ceacam1*. Two founder mice from two embryonic cell clones showed germ line transmission of the targeting construct containing the conditional *Ceacam1* gene. Five mice with homozygous conditional *Ceacam1* alleles were born from these two founder mice. These results suggest that embryonic lethality does not occur in the mice carrying conditional *Ceacam1* knockout alleles, thus it is possible to pursue the function of CEACAM1 in normal prostate development, prostate homeostasis and prostate tumorigenesis *in vivo*.

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Fig. 3. PCR analysis with mouse tail DNA to detect homologous recombination in mice.

-/- +/- +/+

488 b.p. →
382 b.p. →
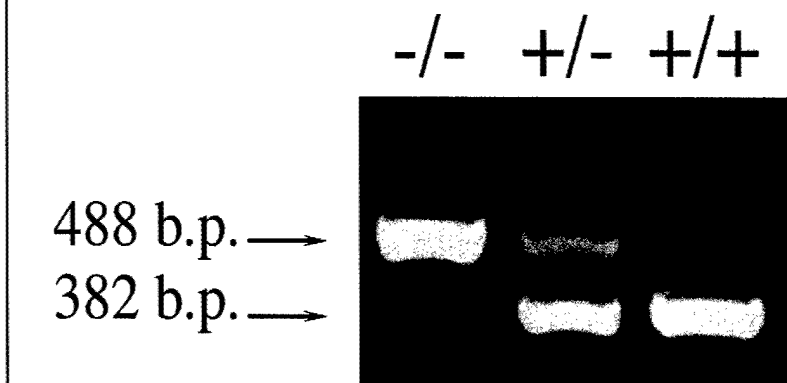
(6) KEY RESEARCH ACCOMPLISHMENTS

- ❖ Designed a knockout strategy that allows Task1 and Task2 to be carried out in parallel.
- ❖ Constructed the targeting vector.
- ❖ Transfected the targeting vector into embryonic stem cells and selected 24 positive ES cell clones.
- ❖ Injected three ES cell clones into blastocysts.
- ❖ Bred and genotyped mice for the presence of recombinant allele.
- ❖ Obtained several mice carrying heterozygote and homozygote recombinant allele.

(7) REPORTABLE OUTCOMES

Han, E., Phan, D., Lo, P., Poy, M. N., Behringer, R., Najjar, S., and Lin, S.-H.: Difference in tissue-specific and embryonic expression of mouse Ceacam1 and Ceacam2 genes. Biochem. J. (2001) 355, 417-423.

(8) CONCLUSION We propose to determine the roles of CEACAM1's growth suppressive activity in prostate growth and tumorigenesis by using gene targeting and embryonic stem cell technologies to generate knockout mice. We have designed and constructed a gene targeting vector for specific disruption of the *Ceacam1* gene. This gene targeting strategy will allow us to perform both straight and conditioned knockout of *Ceacam1* gene in parallel. Embryonic stem cells containing the knockout construct were generated and injected into blastocysts. We have

succeeded in generating mice carrying *Ceacam1* conditional knock out allele. These mice are being examined for *Ceacam1* message and protein levels to see whether the recombinant allele alters the endogenous *Ceacam1* expression level.

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3.   Han, E., Phan, D., Lo, P., Poy, M. N., Behringer, R., Najjar, S. M., and Lin, S.-H. Differences in tissue-specific and embryonic expression of mouse Ceacam1 and Ceacam2 genes. Biochem. J., *355:* 417-423, 2001.

4.   Chang, A. S. Y., Reardon, M. J., and DeMayo, F. J. Germline genetic engineering techniques in endocrinology. Totowa, NJ: Humana Press Inc., 2000.

DAMD17-00-1-0031

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